RESISTANCE TO REINFECTION IN EXPERIMENTAL MOUSE TYPHOID

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(With 2 Figures in the Text)

One of the persistent problems in typhoid fever is the induction of a high degree of immunity by artificial means. The value of vaccination by current methods is still not clear-cut, and it seems probable that recovery from the actual disease confers the highest degree of immunity that man can acquire (Felix, 1951).

In experimental animal studies, the closest parallel to typhoid fever is the mouse disease caused by *Salmonella typhimurium* (Ørskov, Jensen & Kobayashi, 1928), and many workers have used this as a model of the human condition. Here also, survivors of infection have been shown to be more resistant than vaccinated animals to challenge infection (Topley, Wilson & Lewis, 1925; Topley, Greenwood & Wilson, 1931). In most cases the survivors represented only a small proportion of the original herd infected with virulent strains of *Salm. typhimurium*.

These findings have been thought to indicate that heat-killed organisms lack some essential antigen needed to stimulate immunity in the host. Several attempts have been made to improve the quality of the vaccine; e.g. by using alcohol-killed organisms (Schütze, 1941) or purified somatic polysaccharides (Caspar, 1928; Raistrick & Topley, 1934); in general, the results were little better than with heat-killed vaccines.

The bearing of such findings on the nature of immunity in mouse typhoid, and any inferences of practical importance in the design of improved vaccines in typhoid fever must, however, depend on the validity of the comparison between vaccinated animals and survivors of infection. Conventional methods of vaccination (e.g. Greenwood, Topley & Wilson, 1931) involve the administration of two doses equivalent to 100–1000 million organisms, on well separated occasions. Vaccination causes only a few deaths, hence the group of animals given the challenge infection represents virtually all the mice which were immunized. On the other hand, survivors of a previous infection have undergone a radically different experience before challenge. Quantitative studies (Hobson, 1956, 1957*b*) have shown that even small numbers of organisms from a virulent strain of *Salm. typhimurium* establish a progressive infection in most animals with gross bacterial proliferation followed by a slow reduction of bacterial counts in survivors, many of which remain carriers. Thus the total amount of bacterial antigen liberated in the tissues may be much greater than that given in the form of vaccine. Survivors of such infection are more resistant to challenge infection than vaccinated animals (Hobson, 1957*a*), but this may be because they have been exposed to more antigen, not necessarily to better antigens than vaccinated mice.

Furthermore, infection with as few as 10 organisms of a virulent strain of *Salm. typhimurium* results in the death of 50% of the mouse population within 28 days of infection (Hobson, 1957*a*). The survivors could represent either animals which had successfully acquired an immunity during the disease, or an innately resistant fraction of a mouse population selected by the infection. Webster (1924, 1932) and Gowen (1952) demonstrated that large differences in genetic susceptibility to infection existed among mice; resistant strains could be segregated by selective breeding from survivors of infection in accordance with genetic principles. Thus, the different response to challenge of vaccinated animals and survivors of previous infection could be merely a reflexion of a different genetic constitution of the two groups.

In the present experiments, the resistance of vaccinated animals to challenge infection has been compared with that of survivors of infection with a variant strain of *Salm. typhimurium* in which there is no gross overt bacterial multiplication, and in which mortality and natural selection is minimal (Hobson, 1957*b*).

MATERIALS AND METHODS

The methods employed are substantially those described in previous publications (Hobson, 1956, 1957*b*). The virulent organism S 2/446 and its streptomycin-resistant variant S 2/R have also been described previously. As before, all mice were segregated into individual glass jars immediately after inoculation of *Salm. typhimurium*, to exclude cross-infection. The possibility of auto-reinfection from excreta cannot be completely excluded; however, faecal excretion in mouse typhoid is often intermittent or absent (Topley & Ayrton, 1924), and in the present experiments each mouse was changed to a fresh jar at intervals of 2–3 days, so that contaminated food and excreta could not accumulate.

The response to infection was judged by the total specific mortality (proved bacteriologically at post-mortem), by the average time to death, and by determining the degree and frequency of carriage in surviving mice.

At intervals during the experimental infection, mice selected at random were killed and quantitative bacterial counts were made of each organ. In mice infected with the variant strain of *Salm. typhimurium* and subsequently challenged with the virulent strain, the pattern of both infections was followed by performing all bacterial plate counts of organ homogenates in duplicate on nutrient agar and on agar containing streptomycin 200 μg./ml. Equal volumes of tissue homogenate were inoculated in nutrient broth and streptomycin broth, and plated after overnight incubation in all cases where the primary plates showed no growth. The identity of colonies was confirmed by slide agglutination reactions.

RESULTS

The effect of primary infection on intraperitoneal challenge infection

Previous results (Hobson, 1957*b*) showed that in mice infected with 1000 organisms of the relatively avirulent variant strain S 2/R, mortality was low (approximately 13%), and most of the deaths occurred in the first 14 days. Quantitative studies

in mice killed at various intervals after infection showed that the bacterial counts of each organ rose slowly during the first few days, and then remained at a fairly steady level; most animals became persistent carriers.

In the following experiments, mice were inoculated intraperitoneally with 1000 S 2/R organisms; after 4, 12 or 28 days' duration of the primary disease the survivors were reinfected intraperitoneally with 5000 organisms of the virulent strain S 2/446. In each experiment, an equal number of normal mice of the same age and weight, with no previous infection, was similarly challenged. The total period of observation was 28 days; at the end of the experiment all the survivors were killed and examined.

In a group of twenty mice there had been no deaths 4 days after the primary S 2/R infection; these mice and twenty controls were challenged with the strain S 2/446. Twenty-eight days after challenge eighteen (90 %) of the control group of mice had died, and the two survivors were carriers. Only eight (40 %) of the re-infected group died (Table 1, expt. (*a*)); in two mice post-mortem blood cultures showed heavy pure growth of the reinfecting strain S 2/446, and in the other six cases both streptomycin-sensitive (S 2/446) and streptomycin-resistant (S 2/R) organisms were isolated, with a predominance of the reinfecting strain S 2/446. All the twelve surviving mice were persistent carriers of the primary infecting strain S 2/R, small numbers of organisms being found in liver and spleen only. Ten of these mice (83 %) were doubly infected; the total liver and spleen counts varied from 60–3 $\times 10^3$ organisms, with the reinfecting strain S 2/446 predominating in every case.

Table 1. *The effect of previous infection with* Salm. typhimurium *S 2/R or of vaccination with heat-killed organisms on the resistance to infection with* Salm. typhimurium *S 2/446* (500 *bact.i.p.*)

Experiment	Duration of primary S 2/R infection (days) before challenge	No. of mice in each group	Deaths within 28 days after challenge (%)	Percentage of survivors with persistent infection	
				Challenge strain S 2/446	Primary strain S 2/R
(*a*)	4	20	40	83	100
(*b*)	12	20	15	33	100
(*c*)	28	20	5	21	79
(*d*)	Vaccinated mice: 2 weekly doses before challenge	20	65	100	—
(*e*)	Normal mice:* no previous infection or vaccination	80	96	100	—

* The control mice were inoculated in batches of 20 in conjunction with experiments (*a*), (*b*), (*c*), and (*d*).

In a second group of forty mice, only two had died 12 days after S 2/R infection; twenty survivors and twenty normal controls were infected intraperitoneally with 5000 S 2/446 organisms. The result 28 days after challenge is shown in Table 1,

expt. (*b*). Only three mice (15 %) of the previously infected group died in this period, but nineteen (95 %) of the control animals died. All the survivors of the reinfected group continued to carry small numbers of S 2/R organisms, but the reinfecting strain S 2/446 was isolated from only six of the seventeen mice (33 %).

In a third group of mice, two had died by the twenty-eighth day after S 2/R infection; twenty survivors and twenty normal mice were inoculated with S 2/446. The results are shown in Table 1, expt. (*c*). All the control animals died. In the reinfected group only one mouse died, and at post-mortem a heavy pure growth of the reinfecting streptomycin-sensitive strain S 2/446 was obtained from the blood and organs. In thirteen of the nineteen survivors (68·4 %) there was no evidence of superinfection by the challenge strain S 2/446, though the primary infecting strain S 2/R persisted in numbers comparable with normal 56-day survivors. Two mice (10 %) were completely free of infection, and two mice yielded only S 2/446 (streptomycin-sensitive) bacteria in small numbers in liver or spleen. From the remaining two mice both strains were recovered, S 2/R accounting for less than 30 organisms per organ, and S 2/446 for 200–1000 in liver or spleen only.

The resistance of survivors to intravenous challenge

Twenty mice were infected with 1000 S 2/R organisms, and twenty mice of the same batch were kept as controls; 28 days later three of the infected mice had died. The seventeen survivors and seventeen control mice were injected intravenously with 2×10^5 organisms of the strain S 2/446. All the control animals died by the fifteenth day, but sixteen of the seventeen previously infected mice survived to the twenty-eighth day after challenge. Fifteen of the survivors were carriers; eight mice were doubly infected, three were infected only with streptomycin-resistant (S 2/R) organisms, and in the other four the reinfecting strain appeared to have displaced completely the primary strain S 2/R. In all cases the total bacterial count was less than 10^4 organisms.

The duration of resistance to reinfection

Ten mice alive 5 months after infection with 1000 S 2/R organisms were injected intravenously with 2×10^5 S 2/446 organisms. Ten control mice, of the same age and weight, were similarly injected; 28 days later all the control mice had died, but nine of the ten previously infected mice survived. All were carriers of small numbers of both strains of *Salm. typhimurium*.

The resistance of vaccinated mice to challenge

Twenty-five mice were injected intraperitoneally with 1000 million S 2/446 bacilli killed by heating at 56° for 1 hr.; one mouse died with signs of toxicity, and the remaining mice were reinjected with the same dose of vaccine 7 days later. Seven days after the second dose, twenty vaccinated mice and twenty normal controls were injected intraperitoneally with 5000 S 2/446 organisms; 28 days later nineteen of the controls and thirteen vaccinated mice had died (Table 1, expt. (*d*)).

The mean time to death was later in the vaccinated than in the control animals, but the difference in total mortality was not significant (χ^2, with Yates' correction $= 2{\cdot}9$ $P > 0{\cdot}1$), and all the vaccinated survivors were carriers.

The course of a challenge infection in previously infected mice

Forty mice were inoculated intraperitoneally with 1000 S2/R organisms; 28 days later only three (7·5%) had died, and five survivors killed on that day were shown to be carriers of streptomycin-resistant organisms. The thirty-two survivors were inoculated intravenously with 2×10^5 organisms of the virulent streptomycin-sensitive strain S2/446. One mouse died of air embolism; two mice

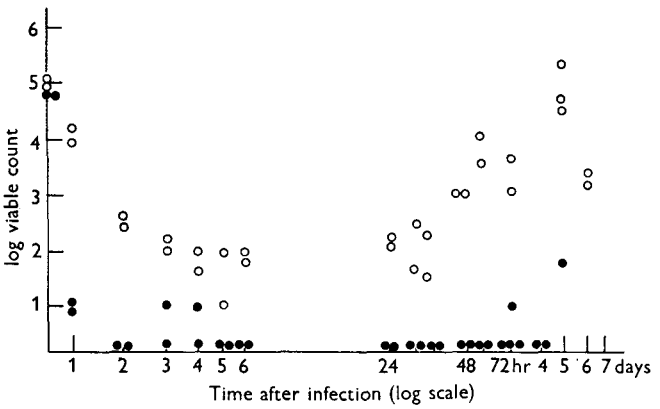

Fig. 1. The rate of blood-clearance of *Salm. typhimurium* S2/446 after intravenous injection (2×10^5 bact.). Bacteria per ml.: ○, normal mice; ●, mice infected 28 days previously with *Salm. typhimurium* S2/R.

were killed immediately after injection; two mice were killed hourly until the sixth hour, and the remainder at intervals over the next 5 days. Bacterial counts of the blood and organs were made on plain media and streptomycin-containing media, to allow the two bacterial strains to be differentiated; thirty of the mice were carriers of small numbers of S2/R organisms. The rate of blood clearance of the streptomycin-sensitive reinfecting strain S2/446 is shown in Fig. 1. The total tissue population (i.e. the sum of blood and organ counts) of S2/446 bacteria is shown in Fig. 2. In a previous experiment (Hobson, 1957*b*) the course of S2/446 infection had been followed in normal mice of the same age and weight. For comparative purposes, bacterial counts in these animals have been repeated in Figs. 1 and 2.

In the previously infected mice, blood clearance was almost completed in 1 hr., and unlike previously normal mice there was no significant reinvasion of the bloodstream subsequently. The total bacterial population fell during the first few hours at approximately the same rate as in animals with no previous infection. During the subsequent period of observation, there was no apparent proliferation of S2/446 bacteria, and the total population remained relatively constant at 10^3–10^4 organisms from the sixth hour to the fifth day. Previous experience has

shown that bacterial counts rose rapidly to levels of 10^7–10^8 organisms over the same period of time in mice with no previous infection.

The course of challenge infection was not investigated in vaccinated mice. Since their resistance to the lethal effect of infection had been shown to be slight, it was felt that any variations in the early course of infection would be too ill-defined for study with comparatively small groups of mice.

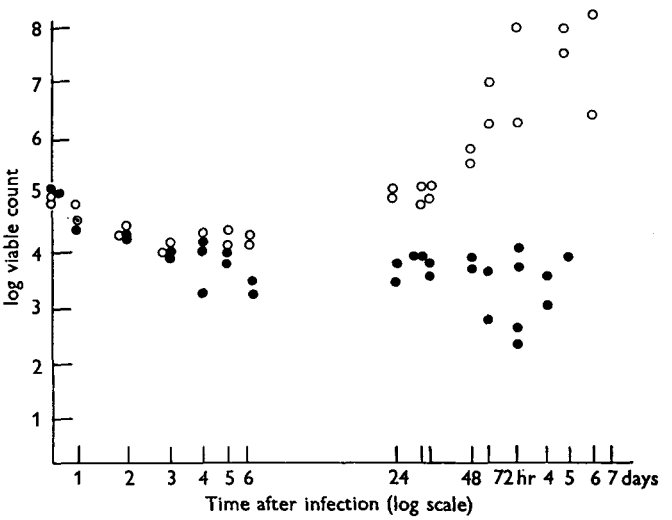

Fig. 2. The course of infection after intravenous injection of *Salm. typhimurium* S 2/446 (2×10^5 bact.). Each point = total population of S 2/446 bacteria in each mouse; ○, normal mice; ●, mice infected 28 days previously with *Salm. typhimurium* S 2/R.

Passive protection from sera from previously infected mice

One hundred mice were infected with 1000 S 2/R organisms and kept in cages of twenty mice each; 28 days later eight mice had died. The survivors were killed and exsanguinated; the pooled serum was stored at $-20°$. A similar pool of serum was obtained from normal mice.

Sixty normal mice were allocated to single jars; twenty were injected subcutaneously with 0·5 ml. of serum from the S 2/R-infected mice, twenty with serum from normal mice and twenty with 0·5 ml. of saline. Two hours later, all the mice were inoculated intraperitoneally with 5000 organisms of the virulent strain S 2/446. On each of the first 5 days after infection, and on the seventh, tenth and fourteenth day the mice were given further subcutaneous injections of 0·5 ml. serum or saline. Twenty-eight days later, the result was as shown in Table 2. The administration of serum from mice surviving a previous chronic infection had no significant protective effect, and all the survivors were carriers.

The distribution of antibody in survivors of S 2/R infection

The serum of sixty-six survivors 28 days after infection with 1000 S 2/R organisms, and thirty-two vaccinated mice, was examined for the presence of O antibody by Dreyer's method (Mackie & McCartney, 1949). Only 16% of the infected

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animals were positive, with a mean titre of 1 in 25; 43% of the vaccinated group were positive with a mean titre of 1 in 37. The serum from ten vaccinated mice and ten survivors of S2/R infection was tested 28 days after inoculation, using the more sensitive method of Felix & Bensted (1954). All sera were positive; the mean titre in the vaccinated group was 1 in 60, and in the infected group 1 in 40.

Table 2. *The passive protection effect of serum from* 28*-day survivors of* Salm. typhimurium *S2/R in normal mice infected with* Salm. typhimurium *S2/446* (500 *bact. i.p.*)

4	4	4	6	7
8	8	9	9	10
11	11	12	12	15
+	+	+	+	+

Serum from previously infected mice

2	2	2	3	4
4	4	4	5	5
5	6	6	7	7
10	10	10	+	+

Serum from normal mice

1	4	4	5	5
5	5	6	6	6
6	6	7	8	8
8	8	9	9	11

Normal saline

Each square represents one mouse, and the number signifies the day of infection when death occurred.

+ = a mouse surviving to the 28th day, with persistent infection.

DISCUSSION

Mice infected intraperitoneally with 1000 organisms of a strain of *Salm. typhimurium* of low virulence have been shown to develop an enhanced resistance to intraperitoneal challenge infection with a virulent bacterial strain. The longer the duration of the primary infection before challenge the lower was the mortality from subsequent challenge infection. Increased resistance to infection was manifest as early as 4 days after infection. The primary and the secondary reinfecting strains of *Salm. typhimurium* could be differentiated in cultures made from mouse tissues, because of their differing streptomycin-sensitivity. Most mice continued to carry small numbers of the primary strain throughout the duration of the challenge infection, but the longer the interval between the primary and the challenge infection, the more mice were capable of eradicating the virulent challenge strain.

The degree of resistance to challenge was greater in these previously infected mice than in mice vaccinated with heat-killed organisms. In vaccinated mice the mean time to death was prolonged, but by 28 days after infection there was no significant reduction in mortality as compared with unvaccinated animals, and none of the survivors had been able to eradicate the infecting organism.

In these experiments, the mortality from the primary infection with the strain S2/R varied from 0–8%. It is a common finding that killed vaccines are liable to cause up to 7% of deaths when used in effective dosage (Greenwood, Topley & Wilson, 1931); in the present experiment one mouse (4%) of the vaccinated group

died during vaccination. Thus, the enhanced resistance of S2/R-infected mice to challenge infection seems unlikely to be due to the effects of natural selection.

Since the group of mice challenged represented virtually the whole original group, their enhanced resistance could only be attributed to characteristics acquired during the primary infection. At first sight, it would appear that immunity had resulted from exposure to small numbers of organisms, since there was no gross proliferation of S2/R bacilli *in vivo*, and the infected mice never contained at any one stage of the infection a bacterial population comparable with the minimum effective vaccine dose. However, infection with *Salm. typhimurium* S2/R was progressive during the first few days after inoculation, and persisted for several weeks thereafter at relatively constant low levels. This finding could only be explained by postulating that bacterial multiplication had ceased but the resting bacteria could not be eradicated, or that bacterial multiplication continued but was accompanied by bacterial destruction by the host, with a constant liberation of antigen not accompanied by any increase in bacterial numbers. Similar hypotheses have been made in experimental tuberculosis, and it was shown by Dubos, Pierce & Schaeffer (1953) that only bacterial strains capable of persistence and multiplication *in vivo* were capable of inducing immunity. It is possible that the constant liberation of antigen in the tissues represents a more physiological stimulus to immunity than the sudden and discontinuous introduction of large toxic doses of bacterial protoplasm. In experimental brucellosis, where killed vaccines are relatively ineffective, previously infected animals have been shown to develop marked immunity to virulent challenge (Pollack, Kelly, Gorelick, Braun & Victor, 1952), and as in the present experiments the paradox of immunity to reinfection, but persistent infection with the primary strain has been observed (Kelly, Gorelick, Silverman & Braun, 1953; Pomales-Lebron & Fernandez, 1953).

The resistance to challenge infection conferred by previous infection with *Salm. typhimurium* S2/R was shown not to depend on any local effect of the primary infection on the peritoneal cavity, since the survivors were highly resistant to virulent challenge by intravenous inoculation.

Quantitative studies on the course of the challenge infection showed that few mice escaped reinfection of some duration. There was little evidence of any rapid bactericidal activity, but bacterial multiplication after clearance from the blood to the tissues was considerably restrained. In general, the behaviour of the virulent strain of *Salm. typhimurium* in survivors of previous infection resembled that of avirulent strains in normal mice (Hobson, 1957*a*). A similar pattern of behaviour was found to occur in experimental mouse tularaemia by Downs & Woodward (1949), and has recently been demonstrated in experimental mouse typhoid by Schneider & Zinder (1956).

The mechanism of the enhanced resistance of previously infected mice is not clear-cut. Survivors of infection were not found to possess higher titres of agglutinins than vaccinated mice of much lower resistance to infection, and there was no significant passive protection of normal mice with serum from immune survivors. Immunity of survivors may be associated less with the production of circu-

lating antibodies than with the development of some form of cellular immunity, as postulated in tuberculosis (Raffel, 1955), or, since most animals continue to carry the causative organism, their enhanced resistance may depend in part on some form of interference, as in virus diseases (Henle, 1950) or on a depression-immunity of the type described by Morgenroth, Biberstein & Schnitzer (1922). These points could be determined only if some schedule of chemotherapy could be devised to eradicate the primary infection after various durations. So far, however, most schedules of treatment which are effective in acute Salmonella infections fail to sterilize established chronic infections (Smith, 1955; Hobson, 1956). The progressive nature of the immunity in infected animals, despite the gradual downward trend of bacterial counts of the primary infection, makes the hypothesis of interference unlikely.

SUMMARY

Mice surviving infection with a strain of *Salm. typhimurium* of reduced virulence developed a progressive resistance to reinfection with virulent strains.

The degree of resistance was greater than that of vaccinated mice, although the primary infection had not caused any significant degree of natural selection or higher serum titres of O antibody.

The previously infected animals responded to reinfection with a virulent strain of *Salm. typhimurium* by gradually restraining bacterial growth. Many of the survivors eradicated the reinfecting strain.

The possible reasons for the difference between vaccinated mice and survivors of previous infection have been discussed.

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